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(54) Antibody serum

(57) Blood grouping serum containing antibodies specific to human blood antigens and capable of direct agglutination of red blood cells without enhancing the spontaneous agglutination of immunoglobulin-coated cells, are produced by treating whole serum or a fraction containing IgG antibodies, separating IgM and IgG, reducing and thereby breaking disulfide bonds in the IgG, fixing the reduced IgG to prevent re-oxidation, and recombining with IgG.

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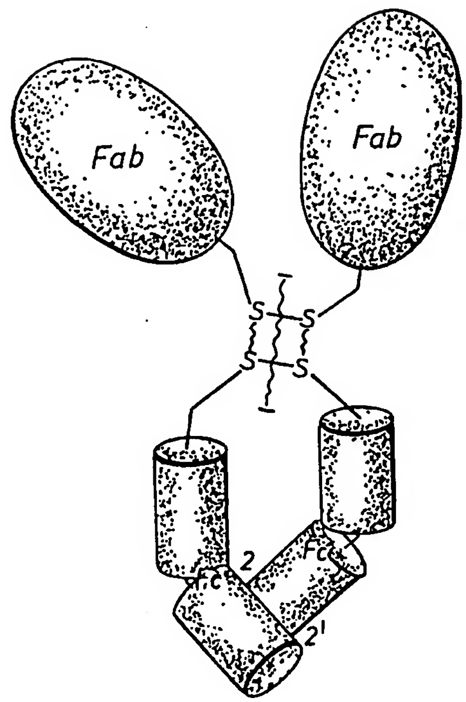
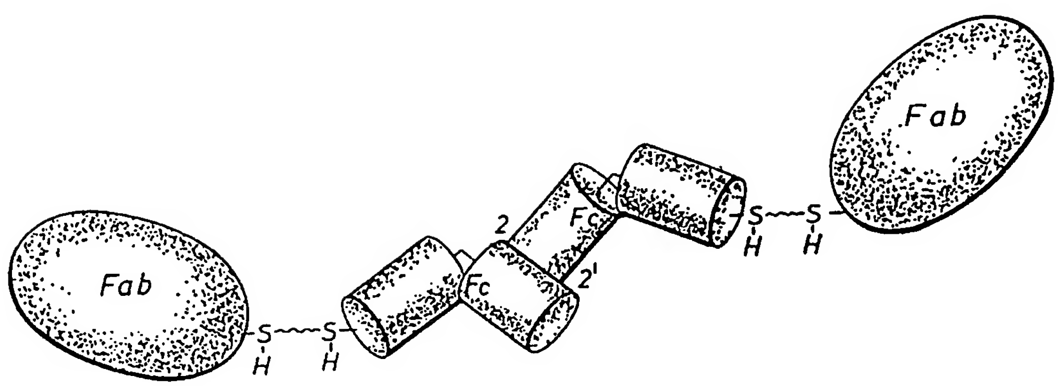


FIG. 1

FIG. 2



SPECIFICATION

Antibody serum

Over three hundred different blood group antigens have been identified in human blood. Not all of these antigens appear in the blood of most humans. However, depending upon a number of factors, some known but many unknown, a combination of such antigens will be found in the blood of every individual. Accordingly, when the blood of one individual is introduced into the circulation of another, as by a transfusion or pregnancy, chances are that the blood of the donor will contain one or more of the antigens not present on the red cells of the recipient. If this occurs, the immune mechanisms of the recipient may respond to the presence of these foreign antigens by producing antibodies in the recipient. Depending upon the number and characteristics of the specific antigens involved, the presence of these antibodies in the blood serum of the recipient will produce effects which may be serious and which must be monitored and otherwise taken into account in subsequent transfusion or pregnancies.

To avoid or, at least, to minimize a potentially detrimental immune response, systems and procedures have been developed for typing blood and for matching donor and recipient bloods before transfusion. Laboratory identification of the most clinically significant antigens on the red cells of blood is typically carried out through the use of commercially available "anti-serums", i.e., blood serums containing known antibodies. The presence of each of the relevant antigens is determined by testing the red cells of the blood to be typed with the required anti-serum by specific test methods. Agglutination of the red blood cells being tested constitutes a positive test and indicates that the antigen corresponding to the anti-serum used is present, while no agglutination constitutes a negative test and indicates that the corresponding antigen is not present. Agglutination takes place through the linkage of the red blood cells as a result of the antibody in the anti-serum combining with antigens on two or more blood cells.

Antibodies are characterized as being "complete" or "incomplete", depending upon whether or not they have the characteristic of producing agglutination of the red blood cells in saline suspension. Antibodies specific to a particular antigen may occur in both forms, usually in varying proportions, depending upon the individual. There is no presently known way to predict, before an individual is exposed to a particular antigen, whether and to what extent the antibodies produced by that individual in response to the antigen will produce agglutination. Typically, more "incomplete" than "complete" antibody will be generated. Commercial suppliers of specific anti-serums may pool the antibody serum from several different donors in order to produce an anti-serum having a suitable agglutinating quality (determined by a combined consideration of avidity, i.e. binding activity of that specific antibody, and titer, i.e., potency).

Antibodies have been further defined by classifying them according to the nature of the immunoglobulin protein molecule which makes up the antibody, the IgM molecule being that which comprises the naturally "complete" antibody, and the IgG molecule comprising the "incomplete" antibody. It is generally thought that the two immunoglobulin molecules are made up of similar basic polypeptides, the significant difference being that, unlike IgM, the IgG molecule is not large enough to produce a connecting bridge between antigen-bearing red blood cells in saline suspension. Thus, anti-serums manufactured from IgG antibodies require dilution in a high protein solution containing macromolecular additives, which makes them prone to false positive agglutination in certain situations, as will be described.

Further investigation has confirmed that the IgG molecules are actually made up of four polypeptide chains, a pair of so-called "light" chains and a pair of "heavy" chains, held together as a molecular unit by disulfide bonds and by non-covalent interactions, as depicted in Fig. 1 of the drawings. It is on the two "Fab" portions of the molecules that the specific antigen binding sites appear, while the "Fc" portion contains no antigen binding sites. It is also thought that whereas the two antigen binding portions of the IgG molecule are separate from each other (being connected only by the disulfide linkage which joins the two chains), the Fc are connected to each other at a second point, represented in Fig. 1 as the line 2–2'.

It has been postulated and shown in the prior art that, under certain reducing conditions, the disulfide linkage joining the polypeptide chains may be broken without appreciably affecting intrachain disulfide linkages and the non-covalent interactions between the fragments comprising the Fc portion. Under such conditions, and with reference to Fig. 2 of the drawings, it is believed that the two Fab portions thereupon move apart, while the fragments of the Fc portion remain joined at line 2–2'. It is thought that the molecule thus attains a span between the two antigen binding sites that is long enough such that an inter-cellular bridge is formed. The formation of these inter-cellular bridges overcomes the mutually repellent forces between the red cells in saline suspension and agglutination is able to occur. Thus, the modified IgG molecule so produced functions, in effect, as a "complete" antibody.

In the drawings:

Figure 1 is a representation of the physical

structure of an IgG molecule as it exists naturally. The representation is intended to illustrate the different character of distinct portions of the polypeptide chains making up the molecule and how those chains are linked together.

Figure 2 is a representation of the same IgG molecule illustrated in Fig. 1, but after it has been subjected to reducing treatment.

According to the invention, blood serum containing antibodies specific to a particular human blood group antigen is first treated to remove and retain any IgM antibody, leaving a solution containing only IgG antibody. This solution is thereafter submitted to mild reducing conditions sufficient to split the disulfide bond linking the polypeptide chains of the IgG molecule while at the same time not sufficient to significantly reduce other portions of the molecule. This reaction is readily reversible due to reoxidation. Accordingly, the modified molecule must be "fixed" to substantially prevent reoxidation. This may be conveniently carried out by treating the solution containing the modified molecule with an alkylating agent, such as iodoacetic acid or iodoacetamide. In practice, any compound capable of reacting with the sulfhydril groups produced by the reduction process and which is not itself oxidized under any of the conditions to which the modified protein molecule may be submitted subsequently during the processing and/or storage procedures will be acceptable.

Following the fixing step, any reducing and fixing agents are removed from the solution containing the modified IgG molecule, by any appropriate method known to the art, and the solution is thereafter combined with that IgM fraction which was previously removed and retained. The total protein and salinity are adjusted to assure that the antibody serum will be of appropriate avidity and potency.

It will be apparent to those skilled in this art that the critical procedure in the method outlined above will be the actual reducing step. The term "mild reducing conditions" is intended to refer to any conditions which cause the interchain disulfide linkages connecting the polypeptide chains of the IgG molecule to split apart. At the same time, the reducing conditions must be such that remaining portions of the molecule are substantially unaffected. In particular, it is important to note that if the reducing conditions are too severe, specificity of the antibody to the desired antigen may be lost. The reducing conditions are affected by such factors as the reducing agent itself, the pH of the system, the concentration of the reactants and the time of the exposure to the reducing conditions.

It will be appreciated that any chemical procedure which has as its end purpose the modification of an antibody protein structure without altering the antibody activity and specificity of the protein molecule must be carried out with extreme care. It is for this reason that the method of the present invention calls for removing any complete antibody fraction from the serum prior to submitting the serum solution to the reducing conditions. Any IgM molecule remaining in the solution will be reduced by the recommended procedure and, as a result, its acitivty and specificity adversely influenced.

Compositions produced according to the method of the present invention possess agglutinating activity previously attained only by the use of a high protein diluent containing certain macromolecular substances to accelerate agglutination. The modified IgG immunoglobulin molecules behave substantially as IgM molecules in their agglutinating activity. Thus, the agglutinating quality of a specific anti-serum may be made to significantly increase. Because no high protein diluent or other macromolecular substance is necessary to attain the desired agglutinating activity, the composite serums of the invention do not enhace spontaneous agglutination of immunoglobulin-coated red blood cells (as commonly found in certain disease states). Moreover, they have the advantage over reagents prepared from serums containing only native IgM antibodies in that they give macroscopic agglutination of antigen-positive cells without extended incubation.

The method of the present invention is applicable to anti-serums to many human blood group antigens. In particular, blood grouping serums have been produced specific to the Rh antigens D, C, E, c, e, CD, DE and CDE, and to such other blood group antigens as Kell, Fy^a and S.

According to the method of the present invention, blood grouping serum capable of producing direct agglutination of human erythrocytes in saline solution are produced. Direct hemagglutination is practical with the compositions of the present invention.

The compositions of the present invention may be prepared from the serum of a single donor known to possess a specific antibody, or may be produced from pooled serum from several donors. Concentration of IgG molecules in the serum may be determined by known procedures, for example, as by single radial immunodiffusion. The IgG fraction and the IgM fraction are first separated. This also can be done by procedures which are routinely followed in the art and typically calls for a partial delipidation by centrifugation after a procedure to precipitate the heavier molecule fraction. In one procedure fractionation and precipitation may be carried out by treating the serum with ammonium sulfate, followed by anion-exchange chromatography on Whatman DE-52 cellulose. In another procedure, the raw serum is placed in a dialysis tubing which, in turn, is submerged in an excess of deionized water. Dialysis causes the salinity of

the serum to decrease to the point where the IgM molecule is no longer soluble. Centrifugation carried out after that point will enable the separation of the precipitated IgM from the soluble IgG.

The Reducing Agent

Any of the milder reducing agents known to be capable of cleaving the S–S bond in polypeptide molecules without having a strong affinity for other bonds and interchain linkages may be utilized. Examples of suitable reducing agents are 2-mercaptoethanol, cysteine, dithiothreitol (DTT, also known as Cleland's Reagent), dithioerythritol (DTE) and mercaptoethylamine. Applicants prefer to use DDT because it has demonstrated less of a tendency to "over-reduce" and therefore lends itself more readily to a controlled mild reduction.

Concentration

Generally speaking, the acceptable concentration range of the reducing agent will depend upon the nature of the reducing agent and, to some extent, the particular nature of the IgG molecule to be reduced. At all times, the critical determinant will be the creation of controlled reducing conditions which will produce the desired cleavage without affecting the activity or the specificity of the antibody. For both DTT and DTE, a concentration within the range of 0.5 to 1.0 grams per liter has been found quite acceptable.

To avoid modifying the specificity of the antibody with any of the mentioned reducing agents, it has been found that strict attention must be paid to maintaining an appropriate pH level in the solution. The preferred range for the pH will be between about 7.8 and about 8.3. The use of a buffer is recommended to stabilize the pH within this range. A particularly recommended buffer is that known to the art as Tris, which is a commercially available combination of tris(hydroxymethyl)aminomethane hydrochloride and tris(hydroxymethyl)aminomethane.

Reaction Time

One important factor in the development of suitable mild reducing conditions involves the manner and time of exposure of the solution to the reducing agent. Generally speaking, the lower the concentration, the longer the time of exposure necessary to accomplish the desired cleavage. If the reducing agent is added directly to the solution, the solution should be promptly stirred and thereafter allowed to stand at room temperature for the appropriate period of time. Alternatively, higher concentrations of the reducing agent may be added slowly to the solution with constant stirring and/or constant bubbling of nitrogen through the solution. It has also been found acceptable to dialyze the solution against a larger volume of a solution containing the reducing agent.

With reference now to Figs. 1 and 2 of the drawings, there is illustrated a diagrammatic representation of the hypothetical extreme in segmental flexibility in an IgG antibody before (Fig. 1) and after (Fig. 2) mild reduction. The antibody molecule represented is shown to contain two disulfide linkages (which would typically be CYS 226 and CYS 229). The antigen-binding regions of the molecule are indicated as Fab (antigen-binding fragment), each of which is connected through a flexible "hinge" disulfide linkage to an Fc (crystalline) fragment. the crystalline fragment is, in turn, comprised of two distinct domains approximatley equal in size and shape. The two lower domains are shown connected at line 2–2' to form a strongly interacting dimer which has been demonstrated to remain unchanged by mild reduction. The upper domains of the crystalline fragment are glycosylated peptides and therefore do not strongly interact. they are shown connected through the double disulfide linkage which is cleaved readily upon mild reduction along the line 1–1'. Apparently, the remaining bonds permit sufficient flexibility after mild reduction to allow the Fab regions and the upper domains of the crystalline fragments to assume the positions shown in Fig. 2.

Note that the interchain disulfide bonds have been chemically reduced to form sulfhydril groups, each sulfur atom having combined with hydrogen. This reaction, however, is readily reversible, occurring almost immediately upon dialysis, for example. Accordingly, following the mild reduction, the modified molecule represented in Fig. 2 must be "fixed" by replacing the hydrogens of the sufhydril groups with chemical groups which are not readily oxidized. One convenient procedure is referred to in the art as "alkylating" which involves replacing the sulfhydril hydrogen with a lower alkyl group. The preferred alkylation agent is either iodoacetic acid or iodoacetamide. It will be readily appreciated that any reagent which effectively prevents reformation of the disulfide bond without altering the antibody activity or specificity will accomplish the desired end result.

The following Examples illustrate the invention.

Example 1–3

The procedure of the present invention was used to reduce several "incomplete" anti-D preparations in an attempt to produce a modified antibody which would yield direct agglutination of D-positive red blood cells in a saline solution.

A pool of 150 milliliters of anti-D serum was obtained and divided into three separate, roughly equal portions. These were treated as follows:

1. 40 ml. of the raw serum was dialyzed in a 35 mm. dialysis tubing for seven hours at room temperature against two liters of deionized water. After seven hours, the serum showed a volume of 46 ml. and was slightly turbid. This volume was centrifuged for thirty minutes at 2,000 rpm in order to precipitate insoluble material, which was then removed and re-dissolved in saline. The remaining serum from the dialysis, now at a volume of slightly under 46 ml., was mixed with an equal volume of a 0.01 M. solution of DTT (dithiothreitol) in a tris (hydroxymethyl)aminomethane buffer (0.1 M.) at pH 8.2. This mixture was allowed to stand at room temperature for one hour. Thereafter, 0.425 g. of crystalline iodoacetamide was added, to a final concentration of 25mM. The mixture was then stirred briefly to dissolve the iodoacetamide and thereafter permitted to stand in the dark for one hour. After this, the solution was transferred to a 35 mm. dialysis casing and dialyzed over night at room temperature in the presence of a large excess of 0.05 M Tris buffer at pH 7.8 and 0.15 M NaCl. 86 ml. of solution was recovered. To this was added the saline solution obtained by dissolving the precipitate from the first dialysis step. The anti-D activity to D-positive red cells in saline of the solution had increased substantially over the activity as measured prior to the treatment.

2. A second portion comprising 50 ml. of the serum pool was mixed with 50 ml. of 0.01 M. DTT in a 0.1 M. Tris at pH 8.2. The mixture was allowed to stand at room temperature for one hour after which 0.462 g. of crystalline iodoacetamide was added and dissolved by constant stirring. This solution, in turn, was left standing for one hour at room temperature and then transferred to a 35 mm. dialysis casing and dialyzed overnight at room temperature against 3.5 liters of a buffer solution consisting of 0.15 M. NaCl and 0.05 M. Tris at a pH 7.8. The recovered volume of 94 ml. was tested to demonstrate a hemagglutinating activity equivalent to an IgM titer significantly higher.

3. The final 50 ml. portion of the serum pool was mixed with 50 ml. potassium phosphate buffer (ionic strength = 0.1) at pH 7.0. While stirring at room temperature, 43 ml. of a 50% polyethylene glycol 4000 solution was added to bring the PEG concentration to 15%. The resultant mixture was stirred for thirty minutes, during which time a precipitate formed. This was collected by centrifugation as in Example 1 above. The precipitate was then re-dissolved in 50 ml. of 0.15 M NaCl solution. The supernatant solution was dialyzed against 3.5 liters 0.15 M NaCl solution at room temperature. After dialysis overnight the solution was centrifuged to remove the small amount of insoluble material and had a final volume of about 55 ml. A biuret protein determination showed the solution to contain approximately 15 mg. protein/ml. To 50 ml. of this solution was added 77.2 mg. solid crystalline DTT, producing a DTT concentration equal to 10 mM. After stirring to assure complete solution, the mixture was allowed to stand for one hour at room temperature. Thereafter, 0.231 g. of solid iodoacetamide was added and after dissolution, the mixture allowed to remain undisturbed at room temperature for one hour. The reduced, alkylated protein was then transferred to dialysis casing and dialyzed overnight against 0.15 M NaCl solution buffered to 7.8 with 0.05M Tris. 46 ml. of solution was recovered, which solution demonstrated an appreciable anti-D activity in saline direct agglutination test.

Following the procedures outlined above, reduced and modified IgG antibodies have been produced which are specific to blood group antigens D, C, E, c, e, CD, DE, CDE, Kell, Fy^a and S. Moreover, the process of the present invention lends itself readily to the production of acceptable anti-serums specific to any of the blood group antigens.

CLAIMS

1. A method of producing an anti-serum, which comprises (a) removing IgM immunoglobulin fraction from a serum containing IgG immunoglobulin in solution; (b) subjecting the thus-treated solution, containing IgG, to reducing conditions sufficient to split the disulfide bonds linking the polypeptide chains of the IgG, substantially without reducing other portions of the IgG; (c) fixing the reduced IgG, thereby substantially preventing reoxidation and reformation of the disulfide bonds; (d) removing any reducing and fixing agents from the solution; and (e) combining the solution with the IgM fraction removed in step (a).

2. A method according to claim 1 wherein the IgM fraction removed in step (a) is dissolved in buffered saline.

3. A method according to claim 1 or claim 2 wherein step (b) comprises dissolving a reducing agent in the solution and maintaining the pH of the solution.

4. A method according to claim 3 wherein the reducing agent is 2-mercaptoethanol, cysteine, dithiothreitol, dithioerythritol or mercaptoethylamine.

5. A method according to any preceding claim wherein step (c) comprises contacting the solution with an agent capable of alkylating the sulfhydryl group.

6. A method according to claim 5 wherein the alkylating agent is iodoacetic acid or iodoacetamide.

7. A method according to any preceding claim wherein step (d) comprises dialysis.

8. A method according to claim 1 substantially as described in any of the Examples.

9. The agglutination product of human erythrocytes bearing the appropriate specific blood group antigen, in saline solution, with

an antibody serum produced by a method according to any preceding claim.

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